

Assay of Reducing End-Groups in Oligosaccharide Homologues with 2,2'-Bicinchoninate

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For the first time, reducing values of homologous series of oligosaccharides have been determined by the 2,2'-bicinchoninate assay. The extent of Cu^{2+} reduction was monitored by spectrophotometric measurement of $\text{Cu}^{+}/2,2'$ -bicinchoninate complexes. Conditions of the assay were optimized so that relatively uniform reducing values were obtained with oligosaccharides derived from starch, polygalacturonic acid, and chitin, regardless of degree of polymerization. The uniform values resulted because members of each oligosaccharide series were oxidized to similar levels beyond the aldonic acid stage, while oxidation was limited to the reducing-terminal residue. This conclusion was based on direct measurements of quantitative loss of paramagnetic copper (Cu^{2+}) by electron paramagnetic resonance spectroscopy.

Several sensitive and precise assays for reducing sugars have been based on redox reactions involving electron transfers from the aldehyde/hemiacetal functionality to metal ions. Reduced cation concentrations are monitored spectrophotometrically as complexes with various chromogenic chelators. Among the best such chelators is the sodium salt of 2,2'-bicinchoninate (BCA).¹ BCA is a stable, water soluble compound highly specific for Cu^{+} , with which it forms a deep-blue complex in alkaline solution. This property has led to superior methods for monitoring reduction of Cu^{2+} to Cu^{+} and forms the basis for methods to determine both reducing sugars (1-6) and proteins (7).

The first applications (1-3) of BCA to carbohydrates were in the detection of reducing monosaccharides in column effluents. The reagent was then applied (4) in a rapid manual colorimetric method for reducing sugar determination. Sensitivity was improved (5) to the low nanomolar range by replacing L-aspartic acid with L-serine as the chelator of the oxidant Cu^{2+} . The BCA

assay for reducing sugars was recently miniaturized using a microsample plate reader (6). The various BCA assays provide distinct advantages over other Fehling-type assays that utilize cupric ion as oxidant. Most notably they are more sensitive, reagents are more stable, fewer manipulations are required, and they are subject to less interference from buffer ions.

To our knowledge, reducing values of higher oligosaccharides have not been measured by the BCA assay. The purpose of this study was to develop assay conditions whereby members of an homologous series of reducing oligosaccharides would yield a narrow range of reducing values. This required that conditions be established under which members of the series are oxidized to a similar degree (i.e., transfer similar numbers of electrons to Cu^{2+}), regardless of dp.

MATERIALS AND METHODS

Reagents. Disodium-2,2'-bicinchoninate was purchased from Pierce. Glucose, cellobiose, and cellotriose were purchased from Sigma, purified oligogalacturonic acids were isolated as described previously (8), and chitooligosaccharides and maltooligosaccharides were kindly provided by Dr. K. B. Hicks. Aqueous stock solutions of each carbohydrate were prepared at 0.25 mM.

Assay solution A. To a solution of Na_2CO_3 (27.14 g, 256 mmol) and NaHCO_3 (12.1 g, 144 mmol) in water (450 ml), disodium-2,2'-bicinchoninate (971 mg, 2.5 mmol) was dissolved. The solution prepared by adjustment to 500 ml with water is stable for a month when kept in the dark at room temperature.

Assay solution B. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (624 mg, 2.5 mmol) and L-serine (631 mg, 6.0 mmol) were dissolved in water (450 ml) and then adjusted to 500 ml. This solution is stable for a month when refrigerated in the dark.

Assay procedure. The assay reagent was prepared daily by mixing equal volumes of assay solutions A and B. Standard reducing carbohydrate solutions were prepared by diluting aqueous 100 μM solutions of each oligogalacturonic acid, maltooligosaccharide, and cello-

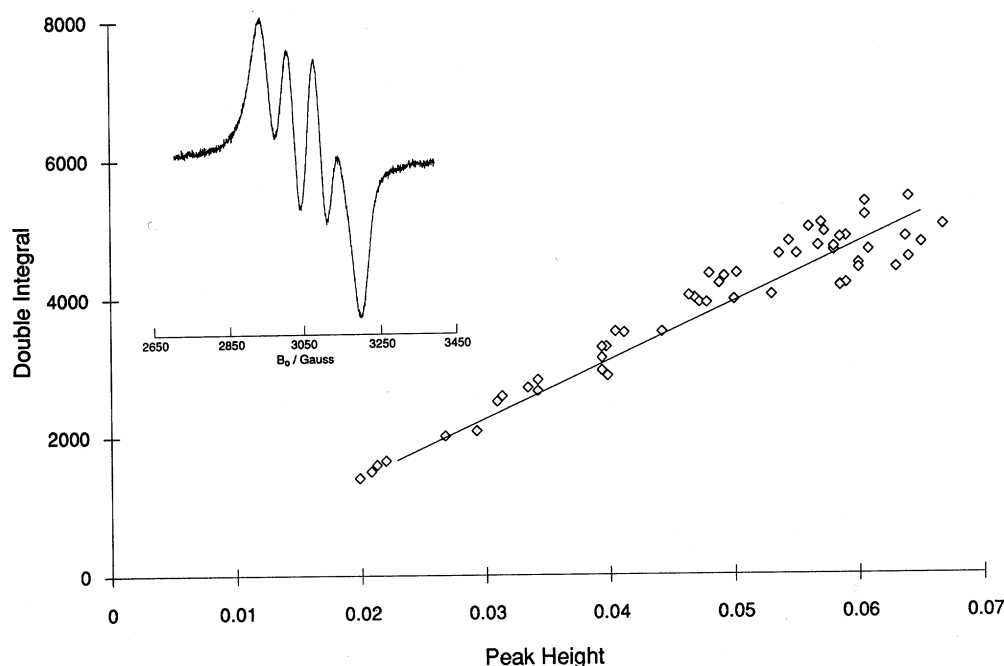


FIG. 1. Changes in the double integral as a function of peak height between the outer transitions in the Cu^{2+} solution EPR spectrum (inset).

biose and cellotriose and by diluting aqueous $250 \mu\text{M}$ solutions of each chitooligosaccharide. Standard curves were obtained with carbohydrate concentrations from 2 to $40 \text{ nmol}/0.4 \text{ ml}$, except for chitooligosaccharides, where the concentration range was from 10 to $100 \text{ nmol}/0.4 \text{ ml}$. To test tubes containing 0.4 ml of reducing carbohydrate, 1.6 ml of assay reagent was added, and the mixtures were heated in a water bath at 80°C for 30 min. Alternatively, 0.1 ml of reducing carbohydrate and 0.4 ml of assay reagent can be used. The tubes are then cooled and read (Perkin-Elmer, Norwalk, CT, Model LC-55 variable wavelength uv-vis detector) at 560 nm against a blank containing no carbohydrate. Absorbance values were constant for several hours.

EPR experiments. Carbonate buffer (with pH 9.2, 9.7, and 10.2; final pH adjustment was made by adding additional aliquots of NaHCO_3) solutions of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and L-serine were as described above for the BCA assay. The initial concentrations of the reagents, however, were such that the molar ratio of Cu^{2+} to galacturonic acid ($2.5 \times 10^{-6} \text{ mol/assay}$) was only 25:1. All mixtures were, except for the controls, heated 5 or 10 min in a water (80°C) bath. All the solutions were quickly cooled to room temperature in an ice:water slurry and electron paramagnetic resonance spectroscopic examination was performed at 20°C in order to directly observe the oxidation of the reducing sugar, D-galacturonic acid, via the quantitative loss of paramagnetic copper (Cu^{2+}). A typical solution Cu^{2+} spectrum is provided in Fig. 1 (inset); in this first derivative plot

($\partial I\{B\}/\partial B$ versus B ; B is the scalar value of the static magnetic field vector, \mathbf{B}) one can clearly see all the components of the electron-nuclear hyperfine interaction of Cu^{2+} 's electron spin with its nuclides ($^{63}\text{Cu}^{2+}$ and $^{65}\text{Cu}^{2+}$; $I = \frac{3}{2}$). A certain amount of *apparent* line broadening is observed only because of the relatively large modulation amplitude (40 G) required to obtain reasonable signal-to-noise. Because of the large amount of reflected microwave power when working with H_2O -based solvents, all experiments were run in capillary tubes containing no more than $10 \mu\text{l}$. General EPR spectral parameters were as follows: scan range, 2000 G ; center field set, 3150 G for Cu^{2+} and 1875 G for the rod-like $\text{Cr}^{3+}/\text{Al}_2\text{O}_3$ crystal; microwave frequency ca. 9.11 GHz ; modulation frequency, 100 kHz ; microwave power, 2 mW ; scan times, 2–4 min, depending on concentration and gain levels; time constant, 8 ms on a Varian Series E-109B spectrometer at 20°C . A ruby crystal (8.99×10^{15} spins) was utilized for the internal calibration of the Cu^{2+} spins via numerical double integration of the first derivative absorption lines (9) after linear baseline correction as follows:

$$\int_{-\infty}^{\infty} \int_{-\infty}^{\infty} \frac{\partial I\{B\}}{\partial B} dB dB \sim \Delta B^2 \sum_{J=1}^N \sum_{I=1}^J \left(\frac{\partial I\{B\}}{\partial B} \right)_I;$$

in the above relationship ($\partial I\{B\}/\partial B$) represents the relative amplitude of each first derivative data point and ΔB the magnetic field interval (0.49 G). Figure 1 demonstrates that there was no paramagnetic dipolar spin-

TABLE 1

Molar Extinction Coefficients (ϵ , 560 nm) of $\text{Cu}^{2+}/2,2'$ -Bicinchoninate Complex Resulting from Assay of Reducing Carbohydrates

Reducing carbohydrate	$\epsilon (\times 10^6)$	Correlation coefficient
Maltooligosaccharides		
Glc	20.5	0.999
(Glc) ₂	19.3	0.999
(Glc) ₃	19.3	0.999
(Glc) ₄	22.5	0.995
(Glc) ₅	23.2	0.998
(Glc) ₇	21.9	0.984
Oligogalacturonic acids		
GalU	14.1	0.998
(GalU) ₂	17.2	0.995
(GalU) ₃	17.0	0.998
(GalU) ₄	18.5	0.998
(GalU) ₅	18.1	0.999
(GalU) ₆	18.6	0.997
Chitooligosaccharides		
Glc _N Ac	6.5	0.992
(Glc _N Ac) ₂	2.8	0.964
(Glc _N Ac) ₃	3.8	0.984
(Glc _N Ac) ₄	3.5	0.949
(Glc _N Ac) ₅	3.6	0.997
(Glc _N Ac) ₆	3.6	0.989

Note. Molar extinction coefficients were calculated from duplicate standard curves for each carbohydrate, which were obtained as described under Materials and Methods.

spin broadening apparent in our experiments since the relationship between the above integral and the first derivative maximum intensity was linear across manifold Cu^{2+} concentrations and pH values.

RESULTS AND DISCUSSION

The BCA assay has not been used to determine reducing values for pure carbohydrates other than the common mono- and disaccharides (4,5). We report values for monomer through heptamer for maltooligosaccharides (Glc_n) and monomer through hexamer for oligogalacturonic acids (GalU_n) and chitooligosaccharides (Glc_NAc_n). The molar extinction coefficients (ϵ) produced by these carbohydrates are listed in Table 1.

Correlation coefficients of regression lines through the standard curve for each carbohydrate (Table 1) indicated excellent agreement in reducing values among the di- and higher oligosaccharides in each series. We found little agreement, however, in reducing values of monomers with those of higher oligosaccharides in the oligogalacturonic acid and chitooligosaccharide series. This discrepancy has been observed using a variety of other reducing end-group assays (10), and we cannot explain these observations. For example, the plot of $\partial A / \partial \text{DP}$ (change in absorbance of the Cu^{2+} /bicinchoninate

complex as a function of DP) against DP of oligogalacturonic acids (Fig. 2) indicates agreement in reducing powers for all oligomers except for the monomer. In addition to the maltooligosaccharides and chitooligosaccharides (Table 1), reducing values for cellodextrins though DP 3 were measured and, not surprisingly, values close to those of the corresponding maltooligosaccharides were obtained. Assay conditions of $80^\circ\text{C}/30$ min were superior to those of $100^\circ\text{C}/15$ min (5), or any other combinations tested, in terms of linearity (to 100 nmol) and comparable ϵ values for all of the oligosaccharides tested. The uniformity of ϵ values among members of a particular oligosaccharide series can be applied to the measurement of dextrose equivalent of starch hydrolyzates. In addition, we routinely apply the BCA assay to measuring the hydrolysis of polygalacturonic acid by endopolygalacturonase, and the assay can be conveniently used in conjunction with other polysaccharide hydrolases.

Most reducing sugars possess different reducing effects upon Cu^{2+} solutions, so even under identical assay conditions, responses to the BCA assay varies somewhat among the various sugars. Exceptions are observed with pairs such as glucose and mannose, which have very similar reducing values (5). At elevated pH, these sugars generate identical enediol and osone structures, which are subject to further oxidation by Cu^{2+} . Such intermediates are generated in alkaline media by all reducing sugars, so Cu^{2+} reduction does not proceed stoichiometrically, nor does oxidation stop at the aldonic acid level with the production of just 2 mole Cu^{+} per mole reducing sugar. From EPR measurements (Fig. 3), we observed that under various pH conditions, up to 17 mole Cu^{2+} were consumed per mole carbohydrate reducing end. As a result, the relative uniformity of reducing values we report is likely due to the fact that the terminal reducing sugars in each oligomeric series are reacting in a parallel fashion. Then, under the assay

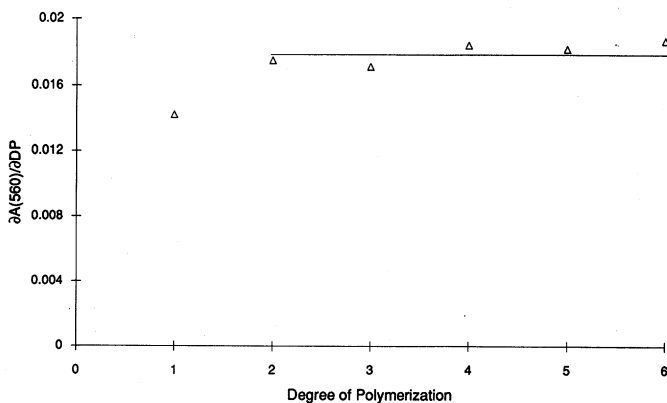


FIG. 2. Plot of $\partial A / \partial \text{DP}$ (change in absorbance of the Cu^{2+} /bicinchoninate complex as a function of DP) against DP of oligogalacturonic acids.

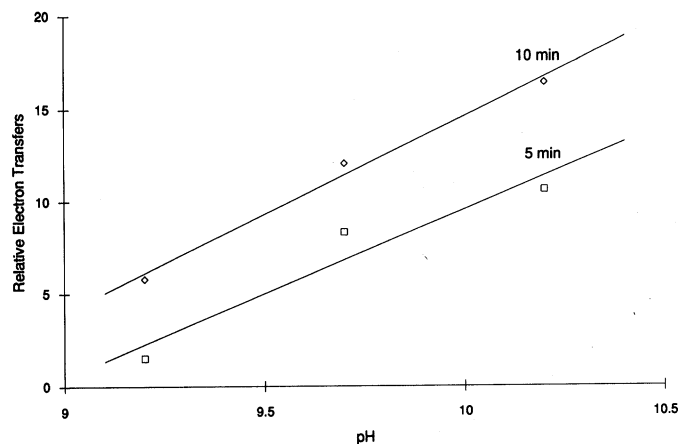


FIG. 3. Effect of pH and different reaction times at 80°C on the transfer of electrons from galacturonic acid to Cu^{2+} as evidenced by change in Cu^{2+} concentration measured directly utilizing EPR spectroscopy.

conditions we describe (80°C/30 min), even though oxidation extends beyond the aldonic acid, it does not proceed beyond the first residue. When the assay is continued beyond 30 min (Fig. 4; for galacturonic acid and oligogalacturonic acids of dp 4 and dp 6), reducing values of the higher oligosaccharides continue to increase after those of the monosaccharides begin to level off. Interestingly, the exponential rate constant, k , from the expression,

$$A = A_0\{1 - e^{-tk}\},$$

describing the relative change in absorbance as a function of time, t , for these differing mono- and oligosaccharides, were virtually identical ($0.0277 \pm 0.0009 \text{ min}^{-1}$).

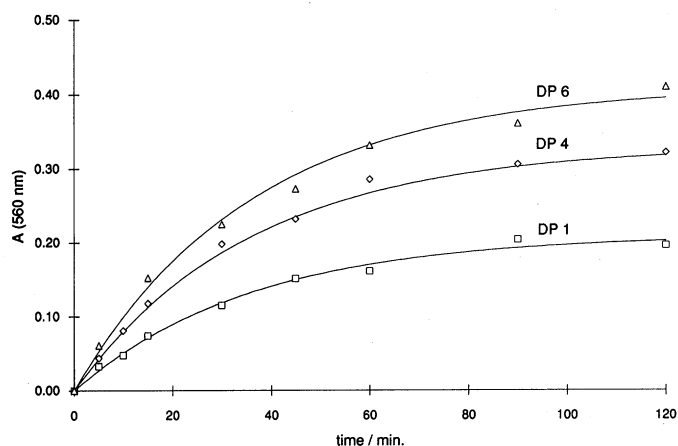


FIG. 4. Change in absorbance at 560 nm of standard BCA assay mixtures as a function of time for various mono- and oligogalacturonic acids. Data were fit to an exponential of the form $A = A_0\{1 - e^{-tk}\}$ utilizing the Gauss-Newton algorithm where k is the exponential rate constant and A_0 is the long duration absorbance asymptote.

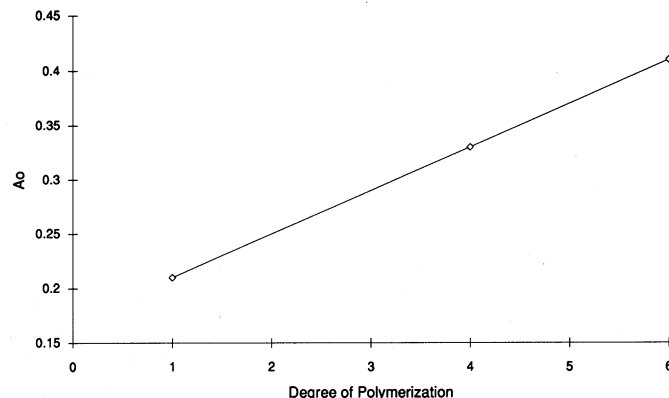


FIG. 5. Relationship of the long duration absorbance asymptote, A_0 , with mono- and oligogalacturonic acid DP.

This latter finding also argues that the initial, or short time, oxidation of the galacturonic acid series does not proceed beyond the first residue since this rate constant should be proportional to the reductant concentration which is obviously related to dp. However, the long duration absorbance asymptote, A_0 (Fig. 5), was found to increase with respect to dp, arguing that the reaction does proceed beyond the first uronosyl residue if the experiment is continued for long time periods (e.g., 135–160 min).

In conclusion, BCA assay conditions have been developed whereby members of homologous oligosaccharide series are oxidized to similar levels, regardless of oligosaccharide size, and therefore possess quite uniform reducing values. In addition, reducing oligosaccharides in multiple samples are conveniently measured. This procedure will allow determination of dp values of oligosaccharides by absorbance comparisons with standards, and kinetic analysis of polysaccharide hydrolases.

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